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RESEARCH PAPER

Activation of PAC_1 and VPAC receptor subtypes elicits differential physiological responses from sympathetic preganglionic neurons in the anaesthetized rat

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BACKGROUND AND PURPOSE
Pituitary adenylate cyclase-activating polypeptide (PACAP) is an excitatory neuropeptide with central and peripheral cardiovascular actions. Intrathecal PACAP increases splanchnic sympathetic nerve activity and heart rate, but not mean arterial pressure (MAP). We hypothesize that the three PACAP receptors (PAC_1, $VPAC_1$ and $VPAC_2$) have different actions in central cardiovascular control, and that their summed effect results in the lack of MAP response observed following intrathecal PACAP injection.

EXPERIMENTAL APPROACH
The effects of the PACAP receptors on baseline cardiovascular parameters were investigated using selective agonists and antagonists administered into the intrathecal space of urethane-anaesthetized, vagotomized and artificially ventilated male Sprague-Dawley rats.

KEY RESULTS
Selective activation of the PACAP receptors had different effects on MAP. When activated by maxadilan, PAC_1 receptors increased MAP. The VPAC receptors decreased MAP when both were activated with vasoactive intestinal polypeptide or when only $VPAC_1$ receptors were activated. The PAC_1 and $VPAC_2$ receptor antagonist PACAP(6–38) had no cardiovascular effects, suggesting that PACAP is not tonically released.

CONCLUSIONS AND IMPLICATIONS
PACAP neurotransmission was not responsible for the moment-to-moment tonic regulation of central cardiovascular control mechanisms. Nevertheless, PACAP release within the spinal cord may have pleiotropic effects on sympathetic outflow depending on the postsynaptic receptor type. PAC_1 and VPAC receptor subtypes produced opposing changes in blood pressure when activated by intrathecal PACAP-38 in the anaesthetized Sprague-Dawley rat, resulting in no net change in MAP.

Abbreviations
HR, heart rate; MAP, mean arterial pressure; PAC_1, PAC_1 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; SHR, spontaneously hypertensive rat; SPN, sympathetic preganglionic neurons; sSNA, splanchnic sympathetic nerve activity; VIP, vasoactive intestinal polypeptide

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), a 38 amino acid excitatory neuropeptide, was first identified in ovine hypothalamic tissue by its ability to stimulate adenylate cyclase. Subsequent immunohistochemical and *in situ* hybridization studies revealed that PACAP was distributed in important cardiovascular regions of the medulla oblongata and spinal cord indicating a possible functional role for PACAP in central cardiovascular control. Physiological studies support these anatomical findings with significant cardiovascular effects reported following central administration of PACAP at different sites in the brainstem and spinal cord (Murase *et al.*, 1993; Uddman *et al.*, 1993; Seki *et al.*, 1995; Krowicki *et al.*, 1997; Lai *et al.*, 1997; Farnham *et al.*, 2008; 2011; Inglott *et al.*, 2011).

A recent study demonstrated PACAP mRNA within bulbospinal C1 neurons of the rostral ventrolateral medulla (>80%) (Farnham *et al.*, 2008), a crucial region in both the tonic and reflex control of the cardiovascular system. The functional significance of these PACAP mRNA-positive bulbospinal C1 neurons was tested by activation of PACAP receptors in the spinal cord, resulting in tachycardia and sympathoexcitation in normotensive and hypertensive rats (Farnham *et al.*, 2011). Paradoxically, the sympathoexcitation was not accompanied by an increase in mean arterial pressure (MAP). Thus far, a mechanism to explain this paradox is lacking. The observed sympathoexcitation was prolonged, and was found in multiple sympathetic beds, so that the MAP effect could not be attributed to differential control of sympathetic outflow (Inglott *et al.*, 2011).

There are three known PACAP receptors: the PACAP-specific, PAC_1 receptor (K_d ~0.5–1 nM), and two other receptors that have an equal binding affinity (K_d ~1 nM) for vasoactive intestinal polypeptide (VIP) and PACAP, the $VPAC_1$ receptor and the $VPAC_2$ receptor (receptor nomenclature follows Alexander *et al.*, 2011). Responses to PACAP in peripheral sites vary, depending on the post-synaptic receptor complement. Here, we test the hypothesis that the different PACAP receptors have different effects on central cardiovascular control. Specifically, that the response to PACAP-38 (from here on referred to as PACAP) depends on the post-synaptic receptor(s) present on different populations of sympathetic preganglionic neurons (SPN), for example, on those chromaffin cells that secrete adrenaline, as distinct from those that secrete noradrenaline (Payet *et al.*, 2003; DeHaven and Cuevas, 2004; Fizanne *et al.*, 2004; Sawmiller *et al.*, 2006; Igarashi *et al.*, 2008). The aims of this study were to determine the contribution of each of the three PACAP receptors, alone, and in combination, on cardiovascular parameters, at the level of the spinal cord. Specifically, the aims of this study were: (i) to determine the involvement of PAC_1 receptors in the intrathecal PACAP response with maxadilan (PAC_1 receptor selective agonist); (ii) to determine the combined involvement of $VPAC_1$ and $VPAC_2$ receptors in the response to intrathecal PACAP using VIP ($VPAC_1$ and $VPAC_2$ receptor selective agonist); (iii) to determine the sole involvement of $VPAC_1$ receptors in the intrathecal PACAP response with PACAP(6–38), the PAC_1 and $VPAC_2$ receptor antagonist and PACAP; and (iv) to investigate the role of PAC_1 and $VPAC_2$ receptors in tonic blood pressure control in the Sprague-Dawley rat using PACAP(6–38). The results demonstrate that PACAP is not involved in the tonic regulation of blood pressure, at the level of the spinal cord. However, activation of the PAC_1 and VPAC receptor subtypes produce opposing effects on blood pressure when activated by their specific agonists/antagonists.

Methods

Animals

All animal care and experimental procedures complied with the guidelines set by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Care and Ethics Committee of Macquarie University. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). We used adult male Sprague-Dawley rats (total = 41; 350–500 g; Animal Resources Centre, Perth, Australia) in these experiments.

General preparation

The animals were housed at the Macquarie University Animal Facility in high-top cages ($n \leq 6$) with environmental enrichment and access to food and water *ad libitum*. The environment was conditioned to have fixed daily 12 h light/dark cycles and temperature was maintained at 21°C ± 2°C. Anaesthesia was induced by bolus injection of urethane (ethyl carbamate; Sigma-Aldrich, Sydney, NSW, Australia; 10% urethane w/v in saline; 1.3–1.5 $g \cdot kg^{-1}$ i.p.). Anaesthetic depth was monitored by observing reflex responses to nociceptive stimuli (e.g. regular paw/tail pinches) and constant monitoring of blood pressure. Additional doses (30–40 mg; i.v.) of urethane were administered to suppress nociceptive or hypertensive reflex responses (>10 mmHg) to pinches. Atropine sulphate (100 $\mu g \cdot kg^{-1}$ i.p.; Astra Pharmaceuticals, Sydney, NSW, Australia) was administered to reduce bronchial secretions. All rats were secured in a stereotaxic frame. The use of a rectal probe, connected to a heating blanket (Harvard Apparatus, Holliston, MA, USA), allowed core body temperature to be maintained between 36.5°C and 37.5°C throughout the experiment.

Surgical preparation

General surgical preparation was carried out on all animals as previously described (Farnham *et al.*, 2008; Gaede *et al.*, 2009; Inglott *et al.*, 2011). The right common carotid artery and external jugular vein were cannulated for the recording of blood pressure and administration of drugs and fluids, respectively. The trachea was intubated to permit artificial ventilation with O_2 enriched air (rodent ventilator; UGO Basile, Biological Research Apparatus, Comerio, Italy) and CO_2 monitoring (Capstar-100 CO_2 analyser; CWE Inc., Ardmore, PA, USA). Heart rate (HR) was derived from an ECG recording. The rats were bilaterally vagotomized, connected to a ventilator and subsequently paralysed with pancuronium bromide (0.8 mg given as a 0.4 mL bolus i.v. followed by an infusion of 10% pancuronium in 0.9% saline at a rate of 2 $mL \cdot h^{-1}$; Astra Pharmaceuticals). The left splanchnic sympathetic nerve was

isolated, dissected and prepared for recording on a silver bipolar electrode (2 kHz sampling, 1 k–100 k × gain, 0.1–2 kHz filtering); a 50/60 Hz line frequency filter (Humbug; Quest Scientific, North Vancouver, BC, Canada) was also used.

An intrathecal catheter (polyvinyl chloride; OD, 0.61 mm; ID, 0.28 mm, Critchley Electrical Products, Australia) with a dead space of 6 μL was inserted into the intrathecal space, through a slit in the dura at the atlanto-occipital joint and advanced caudally to the spinal level of T5/6. All intrathecal injections (10 μL of drug washed in with 6 μL of 10 mM phosphate buffered 0.9% saline (PBS)) were made using a Hamilton syringe (Hamilton Company, Reno, NV, USA) connected to the catheter tubing. All injections were done over a 10 to 15 s period. All rats received control injections of the vehicle, PBS (10 μL of PBS washed in with 6 μL PBS), 30 min before their treatments, as described later.

At the conclusion of the experiments, all rats were killed with potassium chloride (KCl; 0.5 mL; 3 M; i.v.). Post-mortem verification of the location of the intrathecal catheter was achieved by injecting 10 μL of India ink washed in with 6 μL of PBS into the catheter and exposing the spinal cord. The spinal segment level of the catheter was recorded as the level where the tip was observed or where the blue/black spot appeared most intensely on the spinal cord; if the catheter was not found at T5-6, the results were not included in the present study.

Intrathecal drug administration

Role of PAC_1, $VPAC_1$ and $VPAC_2$ receptors in the response to PACAP.

Involvement of PAC_1 receptors. Recombinant maxadilan (from Dr E Lerner) was used as a selective PAC_1 receptor agonist (K_d ~0.5 nM) (Lerner and Shoemaker, 1992; Moro and Lerner, 1997; Vaudry *et al.*, 2009). A dose–response curve to maxadilan was constructed by injecting PBS, and 30 μmol·L^{-1}, 100 μmol·L^{-1} and 300 μmol·L^{-1} concentrations of maxadilan cumulatively (n = 3); responses were observed for 30 min before the next dose was injected. These results were then compared to a 1000 μmol·L^{-1} dose injected intrathecally into a separate group of rats (n = 5; 90 min recording period; the peak responses within the first 30 min post-injection were used for the dose–response curve analysis). The 1000 μmol·L^{-1} dose of maxadilan was excluded from the cumulative dose–response curve due to a limited supply of the peptide.

Involvement of $VPAC_1$ and $VPAC_2$ receptors. A preliminary cumulative dose–response study was conducted to determine the appropriate intrathecal dose of VIP (Auspep, Melbourne, Vic., Australia), the $VPAC_1$ and $VPAC_2$ receptor selective agonist (K_d = 1 nM). Injections of PBS and 30 μmol·L^{-1}, 100 μmol·L^{-1}, 300 μmol·L^{-1} and 1000 μmol·L^{-1} VIP were administered to five rats and responses were observed for 30 min before the next consecutive dose was injected. The 1000 μmol·L^{-1} dose was then administered to a separate set of rats (n = 6); responses were recorded for 90 min.

Involvementof $VPAC_1$ receptors. Rats were pretreated with 1000 μmol·L^{-1} PACAP(6–38) (Auspep), a competitive PAC_1 and $VPAC_2$ receptor antagonist (K_d = 1.5 nM) (Robberecht *et al.*, 1992; Vaudry *et al.*, 2009; Farnham *et al.*, 2011), for 15 min (n = 6) and 30 min (n = 4) prior to PACAP-38 (1000 μmol·L^{-1}; Auspep) administration. Responses were recorded for 60 min following PACAP injection.

In six animals, 1000 μmol·L^{-1} PACAP(6–38) was administered alone; responses were recorded for 30 min.

Data acquisition

Data were acquired using a CED 1401 ADC system (Cambridge Electronic Design, UK) and Spike 2 acquisition and analysis software (version 7.02; Cambridge Electronic Design, UK). The raw data from assays of splanchnic sympathetic nerve activity (sSNA) were rectified, smoothed and averaged to 2 s and normalized to '0' by subtracting residual activity 5–10 min after death. MAP, HR and sSNA were analysed from 5 min blocks taken 10 and 5 min before and 5, 10, 20, 30, 40, 50, 60 and 90 min after intrathecal injections of PBS (up to 30 min), PACAP(6–38) (up to 30 min), maxadilan (up to 90 min), PACAP(6–38) + PACAP (up to 60 min) or VIP (up to 90 min).

Data analysis

Data analysis was carried out in GraphPad Prism software (version 5.03). Summary data are shown as means ± SEM. Statistical significance was determined using one- or two-way ANOVA with Bonferroni's correction, unless otherwise stated. $P < 0.05$ was considered to indicate a significant difference between the means.

Results

Dose–response effects of maxadilan and VIP

Dose–response curves were generated for both maxadilan and VIP (Figure 1), to determine an effective dose for use in this study. The maxadilan dose–response curve was generated by injecting 30, 100 and 300 μmol·L^{-1} concentrations of maxadilan cumulatively into one group of rats (n = 3), and then by injecting 1000 μmol·L^{-1} maxadilan into a separate group of five rats. Maxadilan dose–response curve data were recorded for 30 min. Of the four doses used, only the 1000 μmol·L^{-1} concentration of maxadilan effectively raised both HR and sSNA ($P < 0.0001$; Figure 1). The 300 μmol·L^{-1} concentration of maxadilan also increased sSNA ($P < 0.01$; Figure 1C). MAP was unaffected by any of the maxadilan concentrations used within the first 30 min post-injection (Figure 1A), but was increased at 90 min by the 1000 μmol·L^{-1} dose (results described later). The VIP dose–response curve (Figure 1) was generated by injecting 30, 100, 300 and 1000 μmol·L^{-1} doses of VIP cumulatively in five rats. VIP dose–response curve data were recorded for 30 min. Only the 1000 μmol·L^{-1} concentration of VIP increased HR and sSNA ($P < 0.01$), and decreased MAP ($P < 0.0001$; Figure 1). Therefore, given the similar K_d values of PACAP, maxadilan and VIP (for the specific target receptors), and the dose–response data, the 1000 μmol·L^{-1} concentration of maxadilan and VIP were used in the remainder of this study.

Cardiovascular effects of activation of PAC_1, $VPAC_1$ and $VPAC_2$ receptors in spinal cord

Involvement of PAC_1 receptors. Activation of PAC_1 receptors with maxadilan, a novel PAC_1 receptor selective agonist,

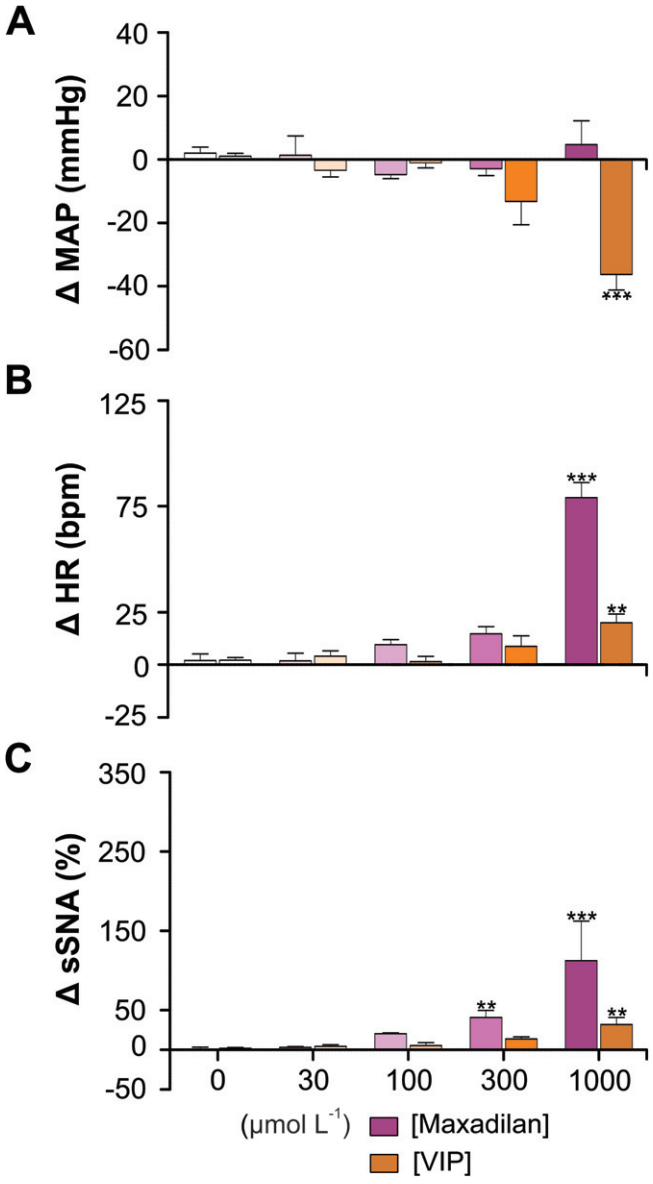

Figure 1

Dose–response curves for maxadilan and VIP. The changes in MAP (A), HR (B) and sSNA (C) before (0 μmol·L^{-1} is a control injection of the vehicle, PBS) and following intrathecal administration of 10 μL of increasing concentrations of maxadilan or VIP. **P< 0.01, ***P< 0.0001, significantly different from PBS.

caused a pressor response after 40 min (Figures 2A and 3), and a prolonged tachycardia and sympathoexcitation (Figures 2A and 3). The responses to the 1000 μmol·L^{-1} dose of maxadilan were recorded for 90 min. Over the 90 min recording period, 1000 μmol·L^{-1} intrathecal maxadilan significantly increased MAP (P < 0.01), HR and sSNA (P < 0.0001) when compared to vehicle (Figures 2A and 3). The HR and sSNA responses to 1000 μmol·L^{-1} maxadilan increased over the experimental time period (Figures 2A and 3), whereas, the MAP response remained unchanged for the first 30 min of response and then increased above baseline after this time (Figures 2A and 3).

Involvement of $VPAC_1$ and $VPAC_2$ receptors. Activation of the $VPAC_1$ and $VPAC_2$ receptors with VIP, the endogenous $VPAC_1$ and $VPAC_2$ receptor agonist, caused hypotension with increases in HR and sSNA. The responses to VIP (1000 μmol·L^{-1}) were recorded for 90 min; there was a significant decrease in MAP (P < 0.0001), but significant increases in HR (P < 0.05; n = 6) and sSNA (P < 0.01; n = 5), when compared to PBS (Figures 2B and 3). HR and sSNA responses peaked ~10–15 min following VIP administration, and remained above baseline for the remainder of the experimental period (Figures 2B and 3). MAP rapidly decreased in response to VIP, reaching its lowest level only 5–10 min after VIP injection. MAP returned to baseline after 50 min and then decreased again for the remainder of the experimental period (Figures 2B and 3).

Involvement of $VPAC_1$ receptors. $VPAC_1$ receptors were activated with an intrathecal injection of PACAP 15 min after intrathecal infusion of the PAC_1 and $VPAC_2$ receptor antagonist, PACAP(6–38). The responses to activation of $VPAC_1$ receptors were recorded for 60 min and caused a significant decrease in MAP (P < 0.01), and significant increases in both HR (P < 0.0001) and sSNA (P < 0.01) when compared to vehicle (Figure 4). All parameters reached a plateau ~10–15 min after $VPAC_1$ activation (PACAP(6–38) + PACAP) and stayed elevated for the remainder of the experimental period (Figure 4). The HR and sSNA responses to $VPAC_1$ activation (PACAP(6–38) followed by PACAP) were significantly (P < 0.05) blunted at all time points, when compared with activation of all three receptors with PACAP alone (Farnham *et al.*, 2008; Inglott *et al.*, 2011) (Figure 5). A separate group of four rats were pretreated with PACAP(6–38) for 30 min before PACAP administration; this did not produce significant attenuation of the PACAP response (data not shown).

In another group of six rats, PACAP(6–38) alone was administered to test the hypothesis that activation of PAC_1 and $VPAC_2$ receptors was involved in tonic blood pressure control in the SD rat. Intrathecal injection of 1000 μmol·L^{-1} PACAP(6–38) had no significant effects on MAP, HR or sSNA when compared to PBS (P > 0.05; n = 6; Figures 4B and 5).

Comparisons of receptor subtype responses

Comparison between individual receptors. When comparing PAC_1 receptor activation (with maxadilan) and that of $VPAC_1$ and $VPAC_2$ receptors (with VIP), the MAP responses were different (P < 0.0001), with PAC_1 receptor activation increasing, and $VPAC_1$ and $VPAC_2$ receptor activation decreasing, this variable (Figure 5). The HR and sSNA responses to PAC_1 receptor activation were greater than those to activation of $VPAC_1$ and $VPAC_2$ receptors (P < 0.0001).

When the results of $VPAC_1$ receptor activation are compared to the results of maxadilan (PAC_1 receptor activation), the MAP, HR and sSNA responses were different (P < 0.0001). $VPAC_1$ receptor activation decreased MAP while that of PAC_1 receptors increased MAP (Figure 5). Activation of PAC_1 receptors induced higher (P > 0.0001) HR and sSNA responses compared to activation of $VPAC_1$ receptors (Figure 5).

When the results of $VPAC_1$ receptor activation were compared to the results of VIP ($VPAC_1$ and $VPAC_2$ receptor activation), only the MAP response was different (P < 0.0001).

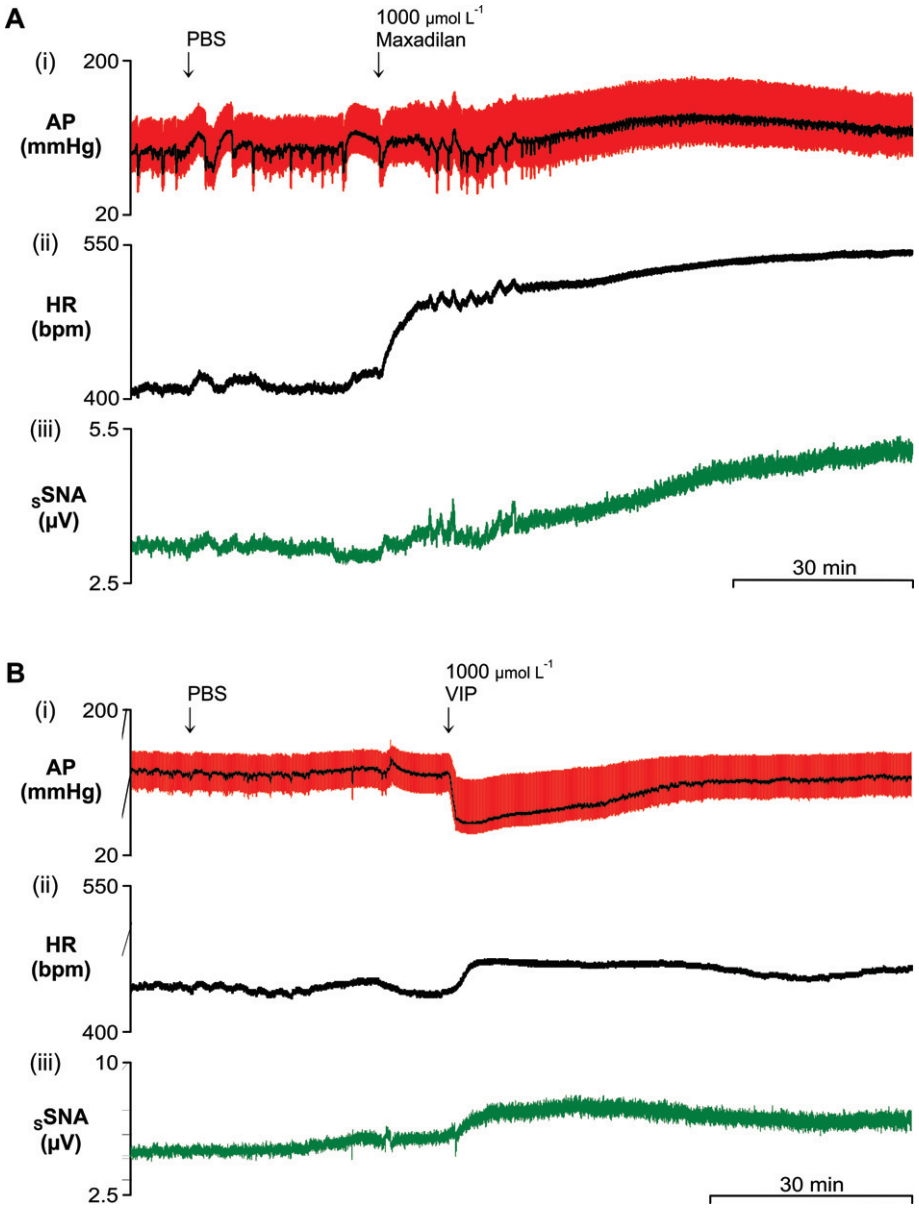

Figure 2

Effects of activation of PAC_1 and VPAC receptors with intrathecal maxadilan and VIP, respectively, on MAP, HR and sSNA. Experimental records show the effects of intrathecal vehicle (PBS) and PAC_1 activation with maxadilan (A), and PBS and VPAC receptor activation with VIP (B) on (i) AP (MAP is represented by the black line on the AP trace), (ii) HR and (iii) sSNA over a 90 min period. Arrows indicate times of PBS, maxadilan or VIP administration.

The decrease in MAP following $VPAC_1$ receptor activation was blunted ($P < 0.0001$) when compared to the MAP response following $VPAC_1$ and $VPAC_2$ receptor activation (Figure 5). There was no significant difference between the HR and sSNA responses to $VPAC_1$ receptor activation compared with those of $VPAC_1$ and $VPAC_2$ receptors ($P > 0.05$; Figure 5).

Individual receptor subtype responses compared to overall PACAP response. PAC_1 receptor activation caused greater changes in MAP, HR and sSNA ($P < 0.0001$) when compared to the previously reported responses to PACAP (Figure 5). Activation of $VPAC_1$ and $VPAC_2$ receptors induced a greater fall in MAP ($P < 0.0001$) and attenuated ($P < 0.05$) the sSNA response, but had no effect on HR ($P > 0.05$) when compared to the responses to PACAP alone (Figure 5).

When comparing the responses of MAP to PACAP alone and $VPAC_1$ receptor activation, PACAP alone did not cause a significant change in MAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011), whereas in this study, we showed that $VPAC_1$ receptor activation resulted in a significant ($P < 0.01$) 20 mmHg drop, in MAP. As stated earlier, HR and sSNA responses to $VPAC_1$ receptor activation were blunted ($P < 0.05$) at all time points, when compared to PACAP alone (Figure 5).

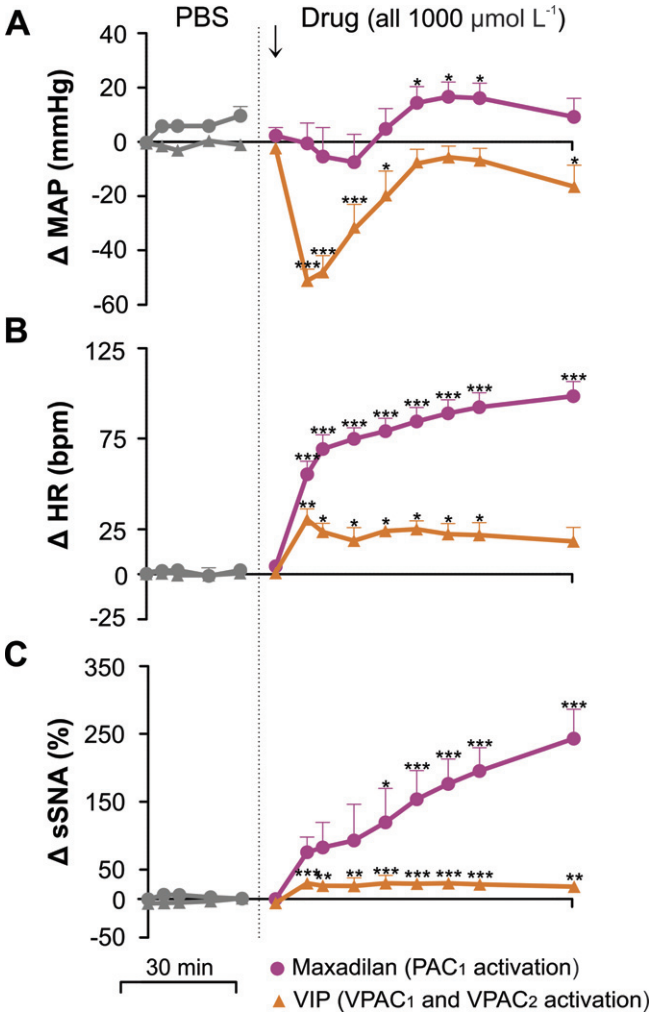

Figure 3

Effects of intrathecal PAC_1 and VPAC receptor activation, with maxadilan and VIP. Time-course changes in (A) MAP, (B) HR and (C) sSNA following 1000 µmol·L^{-1} maxadilan ($n = 5$) or 1000 µmol·L^{-1} VIP ($n = 6$). Maxadilan significantly increased MAP, HR and sSNA, compared with responses to vehicle. VIP caused a significant decrease in MAP, but significantly increased HR and sSNA, compared with responses to vehicle. Arrow indicates time of maxadilan or VIP injection. 'PBS' is the period after intrathecal infusion of vehicle (PBS). 'Drug' is the period after intrathecal injection of maxadilan or VIP. For all; *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, significantly different from vehicle (PBS).

Discussion and conclusions

This is the first study to investigate the pharmacological mechanisms by which intrathecal PACAP exerts potent sympathoexcitatory effects with no corresponding MAP change, and to investigate a possible role for PACAP in the tonic control of blood pressure in the Sprague-Dawley rat (Farnham *et al.*, 2008; Inglott *et al.*, 2011). The novel findings of this study are: first, that the three PACAP receptor subtypes have distinct actions when selectively activated. Activation of PAC_1 receptors increased MAP, while the VPAC receptors decreased MAP. This study is also the first to report the effects of intrathecal infusion of maxadilan (a PAC_1 receptor agonist) and VIP (a $VPAC_1$ and $VPAC_2$ receptor agonist that has no effect on PAC_1 receptors) (Figures 2, 3 and 5). Second, intrathecal infusion of PACAP(6–38) (a PAC_1 and $VPAC_2$ receptor antagonist) exerted no cardiovascular effects, suggesting that PACAP was not tonically released in the spinal cord of the Sprague-Dawley rat (Figure 5), or, if it is, its effects were balanced by other mechanisms. Therefore, we conclude that endogenous PACAP and/or VIP, released in response to a stimulus, may differentially activate PACAP receptors in the spinal cord to modulate efferent sympathetic nerve activity. Following intrathecal infusion of a high concentration of PACAP, all three receptors are activated, so that the sum of these different effects is no overall change in MAP (Farnham *et al.*, 2008; Inglott *et al.*, 2011). We propose as a mechanism, that spinal PACAP receptors are differentially expressed on the SPN responsible for adrenaline or noradrenaline secretion from the adrenal chromaffin cells, and from the SPN regulating the heart and blood vessels (Figure 6).

In this study, we measured responses to intrathecal administration of selective PACAP receptor agonists and antagonists. When compared to previous intrathecal PACAP responses (Inglott *et al.*, 2011), the HR and sSNA responses to PAC_1 receptor activation were augmented significantly, and the MAP was increased by PAC_1 receptor activation (responses to the selective agonist maxadilan). The results demonstrate that PAC_1 receptors primarily mediated the tachycardia and sympathoexcitation observed following intrathecal PACAP administration. Activation of PAC_1 receptors also increased MAP, an increase that was potentially masked by the effects of activating VPAC receptors. We hypothesize that the hypertension following maxadilan injection may be due to PAC_1 receptor-mediated noradrenaline release from adrenal chromaffin cells (Payet *et al.*, 2003).

Our study is the first to report the effects of central administration of maxadilan *in vivo*, in an anaesthetized rat preparation. The results of the present study suggest that when administered centrally, maxadilan, and therefore activation of PAC_1 receptors, has an excitatory effect on SPN, resulting in hypertension mediated by sympathetic vasoconstriction and tachycardia. Although *in vivo* activation of peripheral PAC_1 receptors, with PACAP or maxadilan, induced potent peripheral vasodilation (Lerner *et al.*, 2007; Vaudry *et al.*, 2009), both PACAP and maxadilan increase intracellular cAMP and Ca^{2+} (Grevelink *et al.*, 1995; Jackson *et al.*, 1996; Eggenberger *et al.*, 1999; Pirger *et al.*, 2010), and would therefore be expected to have similar central functions.

Intrathecal PACAP administration activates all three receptors leading to widespread sympathoexcitation, but, surprisingly, no net effect on MAP (Farnham *et al.*, 2008; 2011; Inglott *et al.*, 2011). We propose that this lack of a hypertensive response is due to a balance in the sympathetic efferent effects of PAC_1 receptors, compared with those of activation of $VPAC_1$ and or $VPAC_2$ receptors (Figure 6). This hypothesis is supported by the finding that activation of $VPAC_1$ and $VPAC_2$ receptors with VIP caused a significantly greater decrease in MAP and markedly smaller increases in HR and sSNA, when compared with the effects of PACAP (Inglott *et al.*, 2011). Furthermore, the HR and sSNA responses to activation of $VPAC_1$ and $VPAC_2$ receptors are significantly smaller than those after PAC_1 receptor activation. Finally,

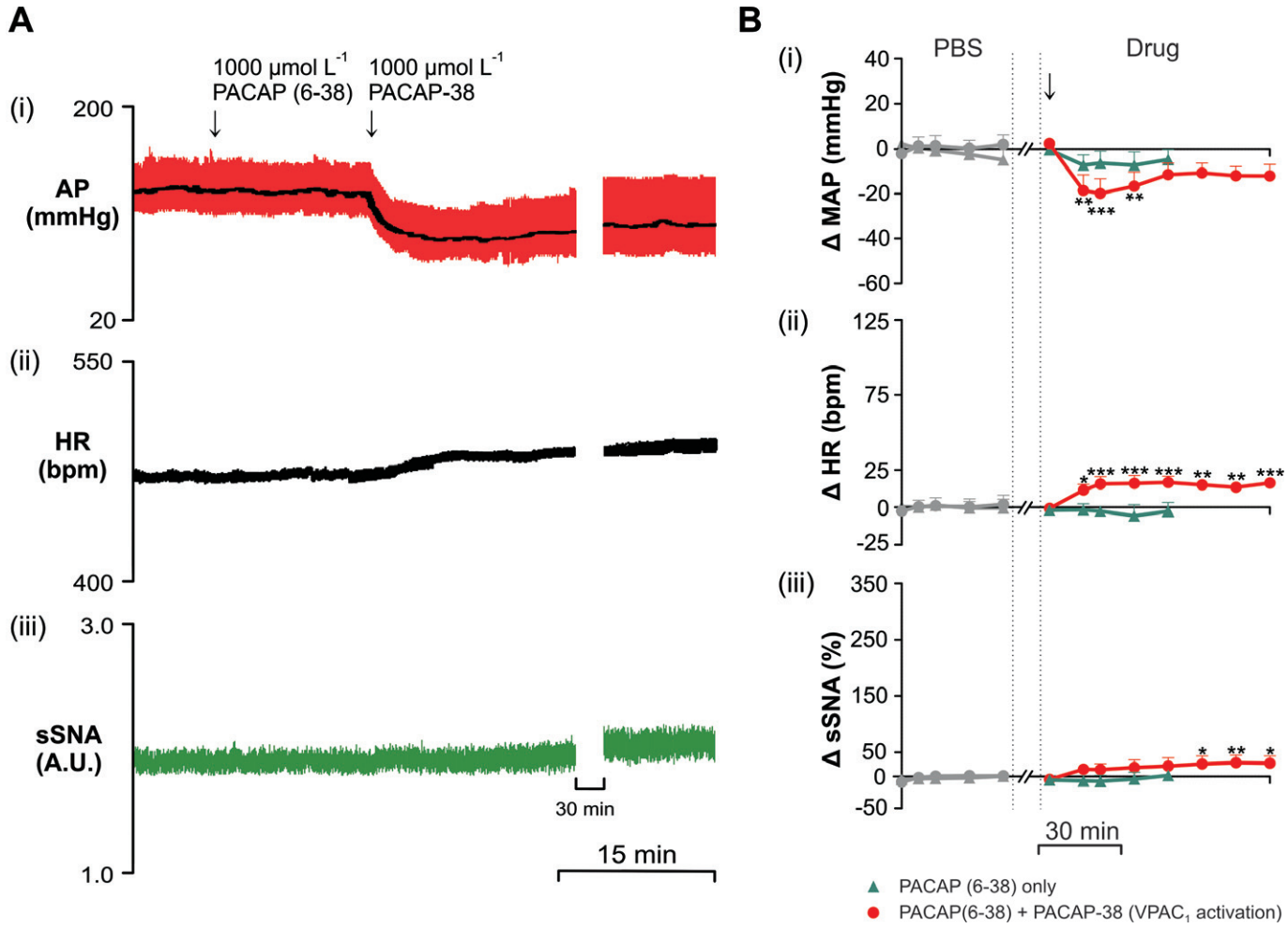

Figure 4

In vivo effects of intrathecal $VPAC_1$ receptor activation with 15 min of PACAP(6–38) followed by PACAP on MAP, HR and sSNA. (A) Experimental record of $VPAC_1$ receptor activation following intrathecal PACAP(6–38) followed by PACAP, on (i) AP (MAP is represented by the black line on the AP trace), (ii) HR and (iii) sSNA over 15 min and 1 h recording periods, respectively. Arrows indicate times of 1000 µmol·L^{-1} PACAP(6–38) and 1000 µmol·L^{-1} PACAP administration. Note the lack of effect of PACAP(6–38). (B) Changes in (i) MAP, (ii) HR and (iii) sSNA after PACAP(6–38) only ($n = 6$) and after $VPAC_1$ receptor activation ($n = 6$). PACAP(6–38) alone does not affect MAP, HR and sSNA compared with responses to vehicle (PBS). PACAP(6–38) followed by PACAP ($VPAC_1$ activation) decreased MAP (Δ $^{-}$20 ± 7 mmHg; $n = 6$), and increased both HR (Δ 17 ± 4 bpm; $n = 6$) and sSNA (Δ 32 ± 14%; $n = 6$). Arrow indicates time of PACAP(6–38) or PACAP injection. 'PBS' is the period after intrathecal infusion of vehicle (PBS). 'Drug' is the period after intrathecal injection of PACAP(6–38) only or PACAP injection following PACAP(6–38). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, significantly different from PBS.

activation of $VPAC_1$ and $VPAC_2$ receptors (with VIP) decreased MAP, while PAC_1 receptor activation (with maxadilan) increased MAP. Thus, the hypotensive response observed following VIP injection suggests that VPAC receptor activation masks the anticipated hypertensive response caused by PAC_1 receptor activation, associated with sympathoexcitation and release of noradrenaline from the adrenal medulla (Payet *et al.*, 2003). We propose that this masking is due to a differential excitation of sympathetic pathways to adrenaline-secreting chromaffin cells (Figure 6), leading to an adrenaline-mediated tachycardia and vasodilatation (Widegren *et al.*, 2010), with a subsequent fall in total peripheral resistance and net hypotension. The concept of differential regulation of chromaffin cells is supported by extensive electrophysiological (Morrison and Cao, 2000) and anatomical data (Kumar *et al.*, 2010).

Previous studies found that intrathecal VIP, administered at the spinal level of T2, did not affect MAP in the rat (Lai *et al.*, 1997), and that intracerebroventricular VIP did not affect MAP or HR in the trout (Le Mevel *et al.*, 2009). Apart from the differences in sites of VIP administration and species, the much lower doses used in these earlier studies may account for the lack of observed MAP and HR responses. Our dose–response data suggest that the dose used in earlier studies would be insufficient to elicit the effects seen here.

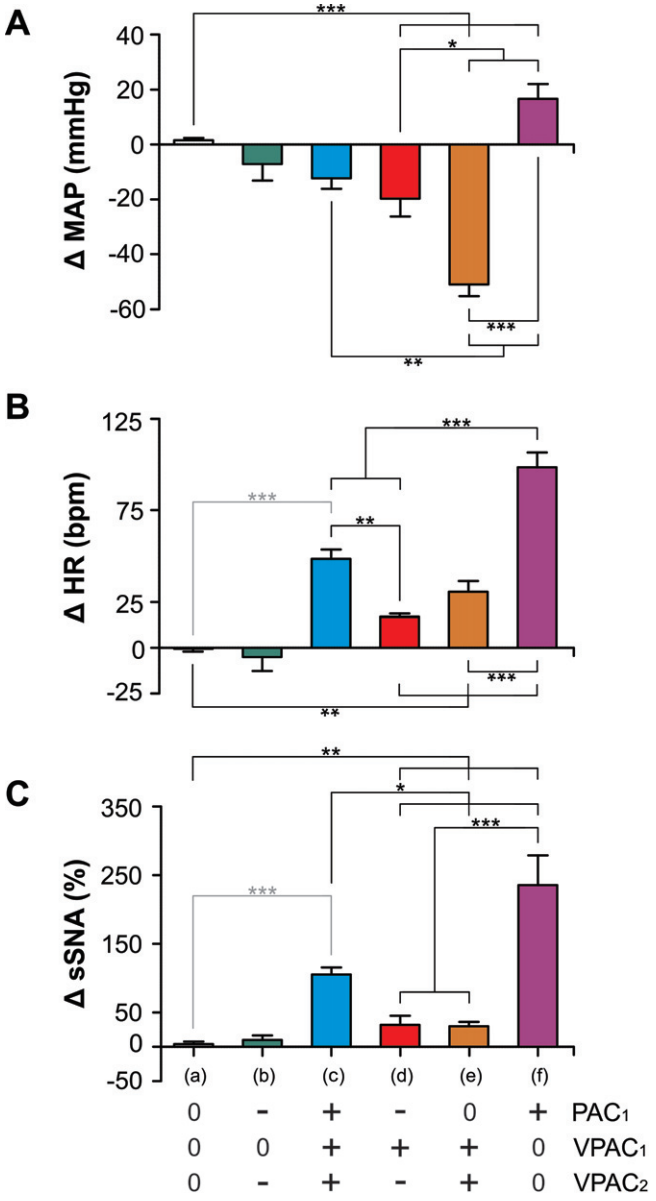

Figure 5

Peak responses following intrathecal activation of the PACAP receptors. Peak changes in (A) MAP, (B) HR and (C) sSNA following differential activation of the three PACAP receptors. In this figure, the treatment groups (abscissa) are as follows; (a) PBS (no PACAP receptors activated), (b) PACAP(6–38) only (PAC_1 and $VPAC_2$ receptors antagonized), (c) PACAP-38 (all PACAP receptors activated), (d) 15 min of PACAP(6–38) + PACAP-38 ($VPAC_1$ receptors activated, other receptors are antagonized), (e) VIP ($VPAC_1$ and $VPAC_2$ receptors activated) and (f) maxadilan (only PAC_1 receptors activated). The PACAP data (treatment group c) is taken from Inglott *et al.* (2011), significance values of these earlier data are shown in grey. The HR and sSNA responses to PACAP (blue) are significantly ($P < 0.05$) attenuated by pretreatment with PACAP(6–38) (red). *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$; significantly different as shown. (+), activated (injection of agonist); (–), antagonized (injection of antagonist); (0), neither activated nor antagonized.

Intrathecal infusion of PACAP(6–38) which is an antagonist at PAC_1, and at $VPAC_2$ receptors but with a 10-fold lesser affinity (Robberecht *et al.*, 1992; Harmar *et al.*, 1998; Dickson and Finlayson, 2009), did not affect MAP, HR or sSNA. This finding indicates that PACAP is probably not tonically released from the spinal cord of Sprague-Dawley rats in this preparation. It is unlikely that $VPAC_1$ receptors compensated for the tonic activity of PAC_1 and $VPAC_2$ receptors in this study, because the $VPAC_1$ receptor response was different from that of the PAC_1 and $VPAC_2$ receptors. The results support our previous study, which concluded that PACAP was not released from the spinal cord of the Wistar-Kyoto or spontaneously hypertensive rat (SHR) (Farnham *et al.*, 2011). Farnham *et al.* (2011) did observe an initial small increase in MAP following PACAP(6–38) injection in the SHR at the same dose. This response is likely to be due to a partial agonist effect that can sometimes occur at high concentrations (Ozawa *et al.*, 1997). The stimulus that evokes endogenous release of PACAP from presympathetic neurons is unknown. PACAP is frequently implicated in responses to stressors such as adrenaline release and haemorrhage (Murase *et al.*, 1993; Kuri *et al.*, 2009).

In summary, PACAP was not tonically released in the spinal cord of the anaesthetized Sprague-Dawley rat and therefore was not involved in the tonic control of blood pressure in this preparation, but could be involved in behavioural responses that require long-term changes in the control of the sympathetic nervous system. The absence of a pressor response following intrathecal PACAP infusion despite a profound sympathoexcitation (Farnham *et al.*, 2008; 2011; Inglott *et al.*, 2011) may be due to a differential activation of SPN that have different complements of PACAP receptors (Figure 6). Finally, activation of spinal PAC_1 receptors alone was sympathoexcitatory and caused a significant pressor response, whereas activation of VPAC receptors resulted in a large depressor response and only mild sympathoexcitation. In conclusion, our data suggest that PAC_1 receptors are part of a sympathetic efferent pathway that is important in the regulation of noradrenaline secretion from the adrenal medulla and control of efferent sympathetic activity to the heart and blood vessels. On the other hand, we suggest that VPAC receptors play a larger role in control of adrenaline secretion. These findings have implications for the management of cardiovascular and metabolic disorders.

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Conflicts of interest

None.

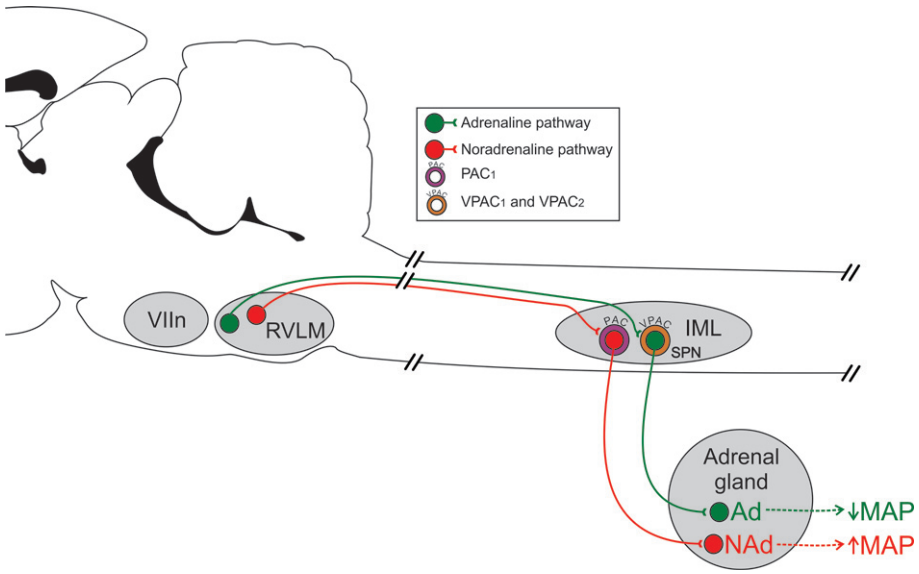

Figure 6

PACAP receptor subtypes differentially regulate catecholamine secretion from the adrenal medulla. Given the findings from the present study, we propose that PAC_1 or VPAC receptors present on SPN differentially regulate noradrenaline- and adrenaline-secreting chromaffin cells. Agonists at PAC_1 receptors activate vasoconstrictor and cardioacceleratory pathways, causing noradrenaline release, a pressor response, and sympathoexcitation. Activation of VPAC receptors present on SPN projecting to adrenaline-secreting chromaffin cells induce adrenaline release, with direct vasodilatory depressor response, mild tachycardia and sympathoexcitation. Ad, adrenaline; IML, intermediolateral cell column of the spinal cord; MAP, mean arterial pressure; NAd, noradrenaline; PAC_1, PAC_1 receptor; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neurons; VIIn, facial nucleus; $VPAC_1$, $VPAC_1$ receptor; $VPAC_2$, $VPAC_2$ receptor.

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